Liposomes coated with hydrophobically modified hydroxyethyl cellulose; influence of hydrophobic chain length and degree of modification

Gro Smistad[a,*], Bo Nyström[b], Kaizheng Zhu[b], Marthe Karoline Grønvold[a], Anne Røv-Johnsen[a], Marianne Hiorth[a]

[a] Department of Pharmacy, School of Pharmacy, University of Oslo, P O Box 1068 Blindern, N-0316 Oslo, Norway

[b] Department of Chemistry, University of Oslo, P O Box 1033 Blindern, N-0315 Oslo, Norway

*Corresponding author:

E-mail address: gro.smistad@farmasi.uio.no

E-mail addresses other authors:

Bo Nyström: b.o.g.nystrom@kjemi.uio.no

Kaizheng Zhu: kaizheng.zhu@kjemi.uio.no

Marthe Karoline Grønvold: marthegronvold@gmail.com

Anne Røv-Johnsen: anne.r.joh@gmail.com

Marianne Hiorth: marianne.hiorth@farmasi.uio.no

Abstract

Nanoparticulate systems with an uncharged hydrophilic surface may have a great potential in mucosal drug delivery. In the present study liposomes were coated with hydrophobically modified hydroxyethyl cellulose (HM-HEC) to create a sterically stabilized liposomal system with an uncharged surface. The aim was to clarify the influence of the amount of hydrophobic modification of HEC and the length of the hydrophobic moiety, on the stability of the system and on the release properties. HM-HEC with different degrees of hydrophobic modification (1 and 2 mole%) and hydrophobic groups with different chain lengths (C8, C12, C16) were included in the study, as well as fluid phase and gel phase liposomes. Both types of liposomes were successfully coated with HM-HEC containing 1 mole% of hydrophobic groups, while 2 mole% did not work for the intended pharmaceutical applications. The polymer coated gel phase liposomes were stable (size, zeta potential, leakage) for 24 weeks at 4 °C, with no differences between the C8 and C16 HM-HEC coating. For the fluid phase liposomes a size increase was observed after 24 weeks at 4 °C for all formulations; the C8 HM-HEC coated liposomes increased the most. No differences in the leakage during storage at 4 °C or in the release at 35 °C, were observed between the fluid phase formulations. To conclude; HM-HEC with a shorter hydrophobic chain length resulted in a less stable product for the fluid phase liposomes, while no influence of the chain length was observed for the gel phase liposomes (1 mole% HM).

Keywords

Phospholipid vesicles; hydrophobically modified; hydroxyethyl cellulose; coating; liposome

Introduction

Liposomes are small, nano-sized vesicles with an inner aqueous core embraced by one or more lipid membranes, usually consisting of phospholipids. They have been widely studied for drug delivery during the last decades, both for parenteral administration, e.g., targeting to tumors or reduction of toxic side effects [1-3], and for topical administration to skin and mucosal membranes [4-6]. The great advantage of liposomes is their ability to encapsulate both hydrophilic and hydrophobic drugs. Some major drawbacks, however, are their short circulation time in the body after parenteral administration and the possibility of chemical degradation, aggregation, and fusion during storage [7-9]. To increase the stability, both *in vivo* and *in vitro*, liposomes have been coated with polymers [10-12]. The polymers forming the coating may be attached to the liposomal surface by electrostatic deposition, e.g., chitosan [10, 13, 14], pectin [15, 16], poly(*N*-isopropylacrylamide (PNIPAAM)-*co*-methacrylic acid) [17], and alginate [18]. Charged polymer coatings have also been introduced for improving the mucoadhesive properties of the system [19-21] and for formulation of pH sensitive [22] and temperature sensitive delivery systems [23].

The stability of colloidal systems is promoted by high surface charge of the nanoparticles [24, 25]. However, charged surfaces may be problematic *in vivo* due to non-intended interactions with biological surfaces and consequently the possibility of toxic effects or precipitation due to interactions with body fluids [26, 27]. In parenteral administration of liposomes an uncharged and hydrophilic, sterically protected surface is a prerequisite for long circulation time [28, 29]. It has also been proposed that nanoparticles with hydrophilic, uncharged surfaces may be advantageous in mucosal drug delivery, since such particles may penetrate deep into the mucosa and deliver the drug close to the cell membrane, instead of being trapped at the surface of the mucin layer [30, 31]. Coating of liposomes without electrostatic interactions is possible by attaching hydrophobic groups to the polymer. During coating the polymer is anchored to the liposome surface by insertion of the hydrophobic groups into the liposome membrane [32-36]. Depending on the type, length, and amount of the hydrophobic groups, the liposome may be stabilized [37, 38] or destabilized and

disintegrating into mixed micelles [36, 37]. Hydrophobically modified chitosan [33, 39], PNIPAAM [23], and alginate [40] as some examples, have been used for liposome coating, in addition to uncharged polymers such as hydrophobically modified poly(ethylene glycol) (PEG) [41], polyvinyl alcohol (PVA) [12] and dextran [37, 38].

Hydroxyethyl cellulose (HEC) is another uncharged polymer. HEC is a cellulose derivative with hydroxyethyl groups randomly distributed along the polymer chain, and has been widely used as thickening agent in pharmaceutical preparations [42], as matrix in controlled-release solid dosage forms [43] and in mucoadhesive patches [44]. In a previous paper we showed that the interaction between HEC and uncharged fluid phase liposomes was very low, or absent [45]. However, HEC can be modified by attaching hydrophobic groups to the hydroxyethyl groups giving hydrophobically modified HEC (HM-HEC). In the previous paper we showed that by using commercially available HM-HEC in appropriate concentrations, both fluid phase and gel phase liposomes were successfully coated [45]. In the present paper we have scrutinized these liposomal systems more closely by using HM-HEC with defined chain length of the hydrophobic groups and defined amounts of hydrophobic modification. The aim was to investigate how the amount of hydrophobic modification of the polymer and the length of the hydrophobic chain influence the coating process, the stability of the coated liposomes, as well as the lipid phase transition temperature and the release of encapsulated substance. Both gel phase and fluid phase liposomes were included in this study. Clarifying the influence of these parameters would be advantageous in designing liposomal systems consisting of uncharged liposomes coated with hydrophobically modified HEC, to obtain uncharged systems with high stability and low possibility for ionic interactions *in vivo*.

Materials and methods

Materials

In this work, a hydroxyethyl cellulose (HEC) sample with the commercial name Natrosol 250 GR (Lot. No. A-0382), obtained from Hercules, Aqualon Division, was utilized as a reference and as the

precursor for the synthesis of the hydrophobically modified analogue (HM-HEC). The degree of molar substitution of hydroxyethyl groups per repeating anhydroglucose unit of this polymer is 2.5 (given by the manufacturer). The molecular weight (Mw = 400 000) of this sample in dilute aqueous solution was determined by intensity light scattering at 25 ^{o}C [46]. The main chemicals for the synthesis of the hydrophobically modified analogue (HM-HEC) such as glycidyl octyl ether (C8), glycidyl dodecyl ether (C12), and glycidyl hexadecyl ether (C16) were all from Aldrich and used as received without further purification. Phosphatidylcholine from soybean (SoyPC) and from egg (EggPC) (Lipoid S PC and E PC S, respectively) and dipalmitoyl phosphatidylcholine (DPPC) were obtained from Lipoid GmbH (Ludwigshafen, Germany). The chloroform used for liposome preparation, sodium dihydrogen phosphate monohydrate, and disodium hydrogen phosphate dihydrate used in the phosphate buffer were all of analytical grade from Merck (Darmstadt, Germany). The tris(hydroxymethyl)aminomethane was from VWR Chemicals (BDH, Belgium), the fluorescence marker 5(6)-carboxyfluorescein (CF), and Triton X-100 were both from Sigma (USA).

Synthesis and characterization of hydrophobically modified polymers (HM-C8-HEC, HM-C12-HEC, and HM-C16-HEC)

The hydrophobically modified hydroxyethylcellulose samples (HM-C8-HEC, HM-C12-HEC and HM-C16-HEC) were synthesized according to our previously reported procedure [47, 48] and the details and characterization of the samples have been described elsewhere [46, 49].

These polymers were further purified by dialyzing against Millipore water for 3 weeks and finally isolated by freeze-drying. Regenerated cellulose with a molecular weight cutoff of about 8000 was utilized as dialyzing membrane. ^{1}H NMR in DMSO-d_6 ascertained the chemical structure and purity of HM-HEC, and the degree of substitution of the glycidyl alkyl ether groups (C8, C12 and C16) were calculated from the peak ratios between the anomeric protons (4.9 ppm) and the methyl protons (0.8 ppm) of the alkyl group. We have successfully synthesized a series of samples with different

length hydrophobic group (C8, C12 and C16) with different degree of hydrophobic substitution 1.0 mole% and 2.0 mole%, respectively.

Preparation of polymer coated liposomes

The liposomes were prepared by the thin-film method as described earlier [15]. In short, the lipids were dissolved in chloroform and evaporated to dryness in a rotary evaporator. The lipid film was further dried under vacuum to the next day in a Christ Alpha 2-4 freeze drier (Christ, Osterode am Harz) to remove organic solvent rests. The aqueous phase (in most cases 5 mM phosphate buffer at pH 6.8) was added to the film at a temperature above the phase transition temperature (T_c) for the lipid, the sample was slowly rotated for 15 min and then swelled for two hours, still at a temperature above the T_c for the lipid. The flask was gently shaken intermittently during the swelling. The samples were kept in the refrigerator to the next day. A Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada) with two stacked 200 nm polycarbonate membranes (Nucleopore®, Costar Corp., Cambridge, USA) was used for size reduction. The liposomes were extruded ten times through the filters at a temperature above the T_c of the lipid. Polymer solutions were made by dissolving the polymer in 5 mM phosphate buffer pH 6.8 by magnetic stirring overnight. All polymer solutions were filtered through 2 µm filters before use. The liposomes (3 mM) were coated by dropwise (~3 ml/min) adding the liposomes (one part) to the polymer solution (four parts) by means of a peristaltic pump (Watson-Marlow 520S IP3, Cornwall, UK) at room temperature during continuous magnetic stirring. The stirring continued for 5 min after all the liposomes were added. Three parallel samples were made of each combination.

When fluorescence marker was encapsulated into the liposomes, an aqueous phase containing 100 mM CF in 60 mM trisbuffer pH 8.0 was used for hydration of the lipid film. The non-encapsulated CF was removed by gel filtration through PD-10 Desalting Columns (GE Healthcare Biosciences AB, Sweden) immediately prior to coating of the liposomes with polymer. The liposomes were eluted

from the column with trisbuffer and diluted to 3 mM lipid concentration immediately after gel filtration with trisbuffer containing 0.35 M sodium chloride to avoid osmotic shock.

Characterization (size, size distribution, zeta potential, transmittance and phase transition temperature)

The intensity mean diameter of the liposomes and the intensity size distribution of the liposome samples were determined by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) with backscatter detection at 173° and at a temperature of 25 °C. The refractive index and viscosity of pure water were used as calculation parameters for all samples. The samples were diluted with filtered (0.2 µm) 5 mM phosphate buffer (pH 6.8) prior to analysis. Each sample was measured in triplicate after an equilibration time of 300 s.

The zeta potential was determined using the same instrument and same dilution as described above. A dip cell for zeta potential measurements (ZEN1002, Malvern Instruments Ltd., Worcestershire, UK) was used for the measurements. The zeta potential was deduced from the mobility by means of the Smouluchowski approximation using the dielectric constant and viscosity of pure water as calculation parameters. Each sample was measured in triplicate at 25 °C.

The transmittance was determined at room temperature, using disposable cuvettes and an Ultrospec II, 4052 TDS spectrophotometer (LKB Biochrom, UK). The wavelength of the incident light was 550 nm.

The lipid phase transition temperature was determined by differential scanning calorimetry (DSC) (DSC 822 Mettler Toledo, Switzerland) using a scanning rate of 2-4°/min in the proper temperature range. Fluid phase liposomes were dissolved in 35 % ethylene glycol in 5 mM phosphate buffer pH 6.8 to avoid freezing of water. Also, the phase transition temperature of the DPPC liposomes was determined by DLS as described by Nicolas et al. [50], using a step-wise protocol in which the

temperature was changed in steps of 0.2 °C in the range 25-50 °C. The sample was allowed to equilibrate for 60 s at each temperature step and then measured in triplicate. The size and derived count rate, in kilo counts per second (kcps), were recorded during the measurements.

Determination of fluorescence marker

The release of the encapsulated fluorescence marker was quantified by using a Wallac Victor^3 1420 multilabel counter (Perkin-Elmer, Boston, USA) (λ_{ex} 485 nm, λ_{em} 535 nm). Calibration solutions of CF and blanks (60 mM tris buffer pH 8.0 containing 0.35 M NaCl) were applied every time and to each plate. The released amount of CF could be measured directly in the solution due to the high concentration and fluorescence quenching of the CF still inside the liposomes. However, for every sample Triton X-100 was also added to disintegrate the liposomes to determine the total amount of CF in the sample. The amount released was determined in per cent of the total amount in the sample.

Storage stability and release studies

Three parallels of each sample were stored in a refrigerator at 4 °C for 24 weeks. At predetermined time points samples were withdrawn from the bottles and analysed for size and size distribution, zeta potential, and leakage of encapsulated CF.

For determination of the liposome release properties three parallels of each sample were stored in a Termaks TS8056 heating cabinet (Termaks, Norway) at 35 °C for 45 h. Samples were withdrawn at predetermined time points and transferred to a microtiter plate for determination of amount CF released as described above.

Results and discussion

The influence of degree of hydrophobic modification of the polymer was investigated by coating EggPC liposomes with HEC with 1 mole% and 2 mole% hydrophobic modifications. Three different chain lengths of the hydrophobic moiety (C8, C12, and C16) and three different polymer concentrations (0.05% (w/w), 0.1% (w/w) and 0.15% (w/w), all in the semi-dilute concentration regime [51], were included in the study. Representative correlation functions and transmittance for the samples coated with 0.1% polymer solutions are shown in Figure 1.

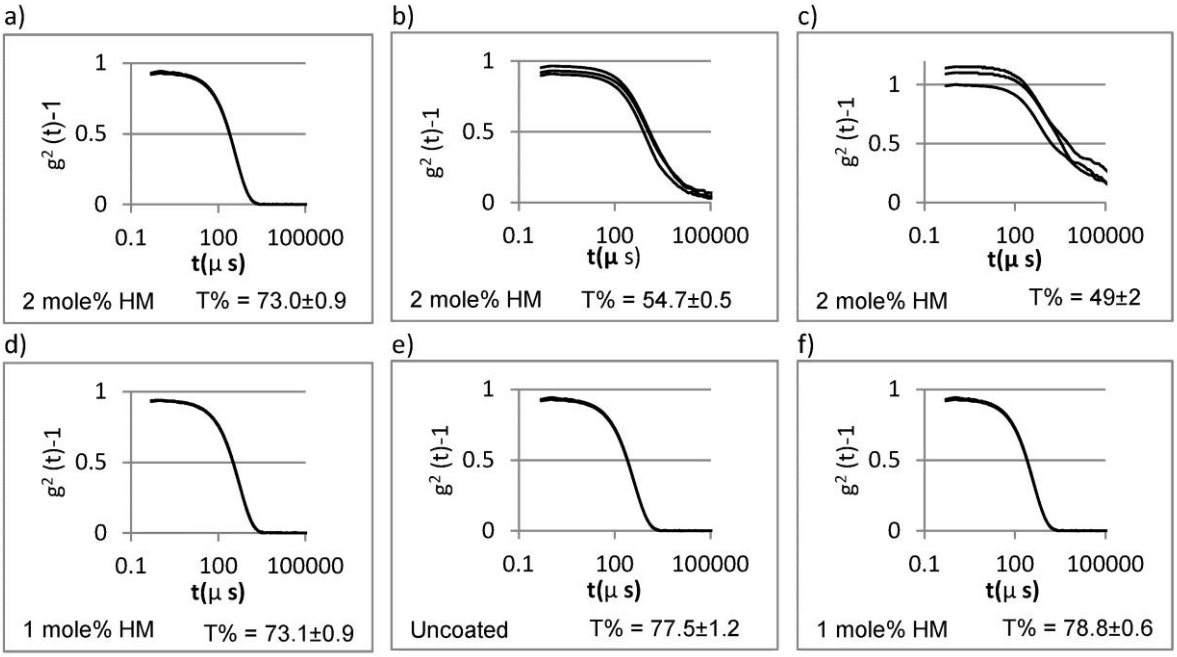

Figure 1

It was not possible to obtain acceptable products when coating with HM-HEC containing 2 mole% of hydrophobic groups, as reflected by two relaxation modes in the correlation functions indicating bimodal distributions. The low transmittance values of the HM-C12-HEC and HM-C16-HEC samples suggest large aggregates and consequently the samples are not suitable for pharmaceutical application. The same trends were observed for the 0.05% and 0.15% polymer concentrations (data

not shown). Coating with HM-C8-HEC containing 2 mole% of hydrophobic groups seemed to result in a more uniform product compared to coating with HM-C12-HEC and HM-C16-HEC (compare the correlation functions in Figure 1), but after one week of storage in the refrigerator, the size distribution of the HM-C8-HEC coated liposomes was clearly bimodal and the product not acceptable for use.

The liposomes coated with HEC containing 1 mole% hydrophobic modification, however, showed monomodal size distributions (as shown in Figure 1) with relatively low PDI values (0.19 and 0.105 for the HM-C8-HEC and HM-C16-HEC coated liposomes, respectively) for the 0.1% polymer concentration. The same trend was observed for the 0.05% and the 0.15% polymer concentrations (data not shown). The sizes of the coated liposomes were larger compared to the uncoated liposomes (increasing from 164 nm for the uncoated liposomes to 347nm and 271 nm for the HM-C8-HEC and HM-C16-HEC coated ones, respectively) but no aggregates or precipitates were observed. It was concluded that 1 mole% seemed to be a proper degree of hydrophobic modification of the polymer for coating the liposomes, both for HM-C8-HEC and HM-C16-HEC, whereas 2 mole% of hydrophobic groups did not produce particles with potential as carriers for drug delivery application due to the heterogeneity of the product. This may be explained by stronger inter- and intramolecular hydrophobic associations at higher degree of hydrophobic modification [52] and consequently the chains are less available for interaction with the liposomal membrane. Furthermore, a long hydrophobic tail has been shown to give stronger intramolecular interactions compared to a shorter one in dilute solutions [52]. Less available hydrophobic groups with the possibility of stronger hydrophobic interactions with the liposome membrane will most probably result in inhomogeneous products. This is also apparent from the correlation functions and the lower transmittance of the HM-C16-HEC (2 mole%) coated liposomes compared to the HM-C8-HEC (2 mole%) coated ones, with the HM-C12-HEC coated liposomes in between (Figure 1).

To study the influence of the chain length on the hydrophobic moiety in combination with the lipid phase on liposome characteristics and stability, two different types of liposomes (SoyPC and DPPC, i.e. liquid-crystalline and gel phase, respectively) were coated with HM-C8-HEC or HM-C16-HEC, both with 1 mole% hydrophobic groups. The polymer concentration chosen for coating was 0.1 % since this concentration appeared as suitable in the preliminary experiments and has also been shown to be proper for coating of DPPC with commercially available HM-HEC in an earlier study [45]. In Table 1, the characteristics of the uncoated and the polymer coated liposomes are shown. It was verified that the scattering intensity of the pure polymer solutions diluted in the same way as the samples was very low compared to the scattering intensity of the samples, which means that the sizes recorded were the sizes of the polymer coated liposomes and not of polymer aggregates.

Table 1: Characteristics of liposomes coated with 0.1 % (w/w) solution of HM-C8-HEC and HM-C16-HEC containing 1 mole% of hydrophobic groups (n=3)

Sample	**Z-Average (diameter)**	**PDI**
SoyPC uncoated	166 nm ± 3	0.07 ± 0.02
SoyPC+HM-C8-HEC	377 nm ± 4	0.20 ± 0.01
SoyPC+HM-C16-HEC	283 nm ± 3	0.11 ± 0.02
DPPC uncoated	1167 nm* ± 75	0.36 ± 0.06
DPPC+HM-C8-HEC	368 nm ± 4	0.22 ± 0.02
DPPC+HM-C16-HEC	267 nm ± 3	0.08 ± 0.02

* Aggregates consisting of liposomes with Z-Average of 161 nm

As can be seen from Table 1, the PDI values are relatively low for all samples. All samples had a zeta potential close to zero (in the range -0.42 to -2.25 mV). The average size of the uncoated SoyPC liposomes is as expected when using 200 nm pore size for extrusion. The uncoated DPPC liposomes, however, appear as very large. This is probably due to aggregation since it has been shown by cryo-electron microscopy that uncoated DPPC liposomes appear as aggregated already one day after extrusion [53]. The aggregation tendency may be due to the partly dehydrated gel phase lipids [54], and consequently relatively low repulsive hydration forces between the liposomes [55, 56]. The DPPC

liposomes in the gel phase have a faceted polyhedral appearance [53, 57], which may facilitate the aggregation due to larger contact areas. When heating the DPPC liposomes to above the $T_{c,}$ the liposomes become more round [17, 57] and the liposomes are expected to disaggregate due to thicker hydration layer and stronger repulsive forces [55, 56], and maybe also because of the smaller contact areas as a result of the spherical shape. In order to determine the "real" size of the DPPC liposomes the sample was first heated to 50 °C (above the T_c of the DPPC) to promote disaggregation. The temperature was then reduced by 0.2 degrees/min and the size was measured at each temperature step. In Figure 2 the sizes and PDI values are plotted against the temperature.

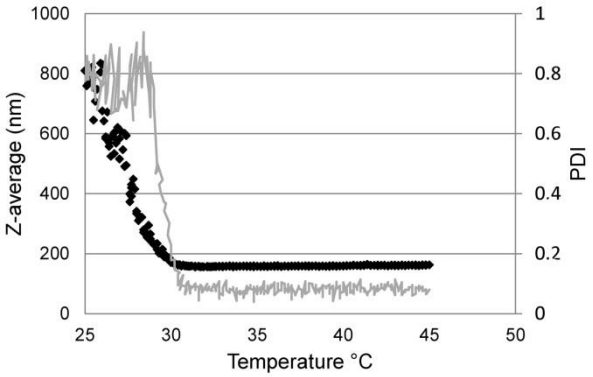

Figure 2

As shown in the Figure 2, at the higher temperatures the size distribution was monomodal (low PDI) and the average liposome size was small (161 nm). It has been shown that DPPC liposomes in the gel phase appear as single liposomes shortly after extrusion [53]. Since the DPPC liposomes in the present work were coated immediately after cooling to room temperature after extrusion, this suggests that the size of the DPPC liposomes at the time of being coated most probably was around 161 nm, i.e., at the same level as the SoyPC liposomes. This means that both the SoyPC and the DPPC liposomes increased in size after coating, which is also in line with what has been shown for other systems in the literature [12, 15, 17, 39, 58]. For both types of lipids the HM-C8-HEC coated liposomes ended up larger than the corresponding HM-C16-HEC coated samples and with larger PDI

values (Table 1). This may suggest that the HM-C8-HEC polymer coating is less firmly attached to the liposome membrane compared to the HM-C16-HEC coating giving larger average particle size.

The stability of the samples was investigated by storing the formulations for several weeks in the refrigerator at 4 °C, and withdrawing samples for analysis at several time points. In Figure 3 the average sizes and the PDI values of the size distributions are shown.

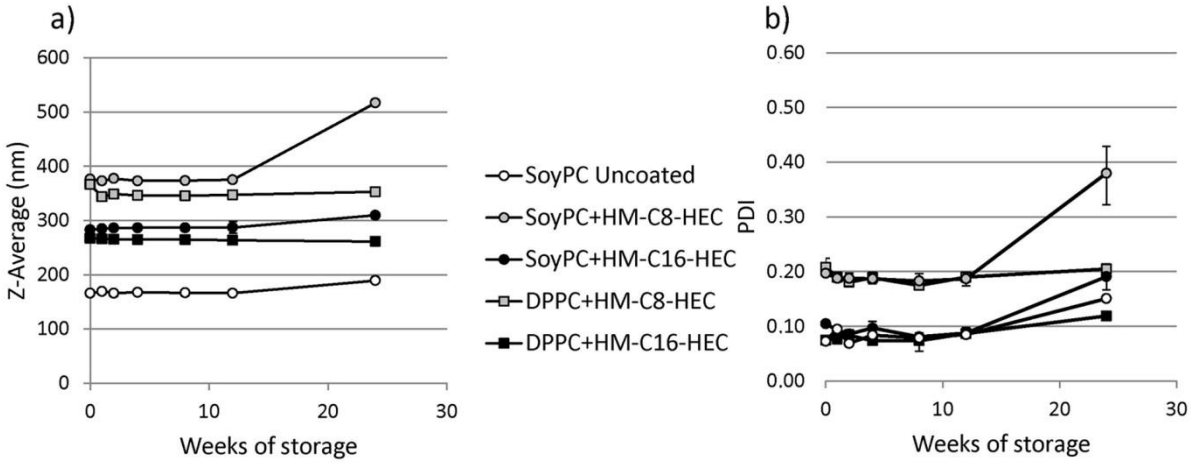

Figure 3

The size of the DPPC liposomes coated with both HM-C8-HEC and HM-C16-HEC remained unchanged during the storage period. The SoyPC liposomes were stable for 12 weeks. However, after 24 weeks significant increase in both the average size and PDI values were observed. The HM-C8-HEC coated liposomes appeared as less stable than the uncoated SoyPC liposomes with an increase in diameter from 375 to 517 nm and an increase in the PDI value from 0.19 after 12 weeks to 0.38 after 24 weeks of storage. Again this may indicate a looser attachment of the HM-C8-HEC coating. Due to the high mobility in the fluid membrane of the SoyPC [59], it is reasonable to assume that shorter hydrophobic chains may detach more easily and interact with other liposomes resulting in bridging between the liposomes as time evolves. Another possibility is that partly detached polymer chains may interact with each other via “hydrophobic micelles” and link the liposomes together, as

described by Chieng and Cheng [34]. Both mechanisms will give larger Z-average values and broader distributions (larger PDI values). The better stability of the DPPC/HM-C8-HEC liposomes may be explained by the ordered gel phase of the lipid membrane with more restricted mobility [35]. The sizes of the uncoated DPPC liposomes were in the range 1100-1600 nm (data not included in the figure due to the large sizes) and remained large during the whole storage period. The smaller and relatively constant size of the polymer coated DPPC liposomes clearly confirms the stabilizing effect of the polymers and is in line with earlier observations [45]. Since the surface charge of the systems was close to zero, the stabilizing effect is most probably due to steric protection.

Lipids in solution may be subjected to chemical degradation, both by hydrolysis and oxidation [8]. Both types of degradation may lead to a more negative zeta potential of the liposomes. In Figure 4, the zeta potential values of the formulations during the storage are shown.

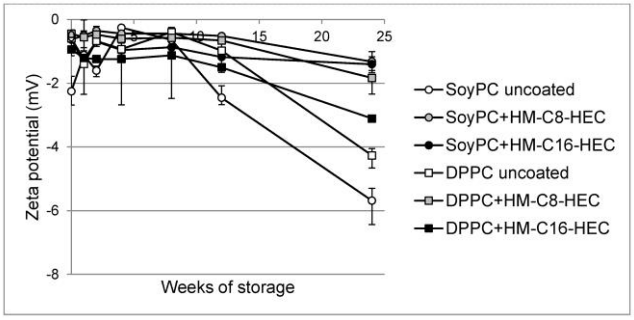

Figure 4

All formulations were slightly negatively charged. The zeta potential remained relatively constant during the storage period for the polymer coated liposomes. For the uncoated liposomes, however, a change to slightly more negative values after 24 weeks of storage was observed. Although the differences in the zeta potentials are small, this may indicate that the liposomes are protected against chemical degradation by the polymer, which is also in line with what has been observed previously for polymer coated EggPC liposomes [45].

Another stability issue of liposomes is loss of encapsulated material. In the present study the leakage of encapsulated CF was tested during storage at 4 °C for 24 weeks (Figure 5).

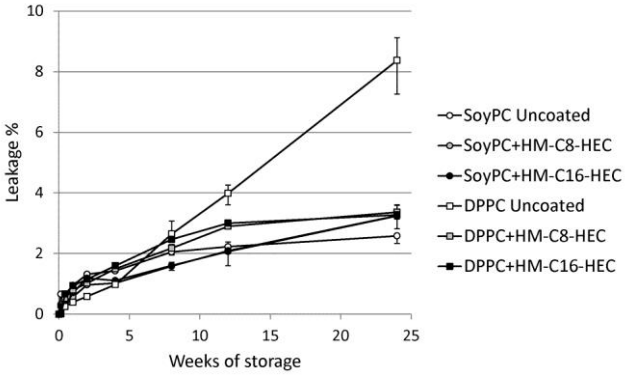

Figure 5

For all formulations, except the uncoated DPPC liposomes, the leakage was very low and less than 4% after 24 weeks of storage. There were no significant differences in leakage between these formulations. The leakage from the uncoated DPPC liposomes, however, was significantly higher, but still less than 10 % after 24 weeks. The higher value can be explained by the aggregation of the DPPC liposomes, since close contact between the liposomes for weeks may be expected to promote fusion and leakage. A close contact between the vesicle membranes of the polymer coated DPPC liposomes was avoided due to the steric stabilization. Low leakage during storage at 4 °C has also been reported for positively charged liposomes coated with negatively charged polymers [11]. However, coating of neutrally charged liposomes with positively charged polymers has been shown to increase the leakage [19, 20, 60], probably by creating pores in the liposome membrane due to interaction between the positively charged groups and the negative charge of the phospholipid [60].

A possible field of application of polymer coated neutrally charged liposomes could be mucosal administration in the oral cavity. The oral mucosal temperature has been reported to be in the range 35.5-36.5 °C and dropping when the mouth is opened [61]. Thus, to investigate the influence of the

polymer coating on the release of encapsulated material from the liposomes, the experiments in the present study were chosen to be carried out at 35 °C. The results are shown in Figure 6. There was a significant difference in the release rate between the SoyPC and the DPPC formulations; the DPPC formulations showing a very low release, less than 1% after 45 h.

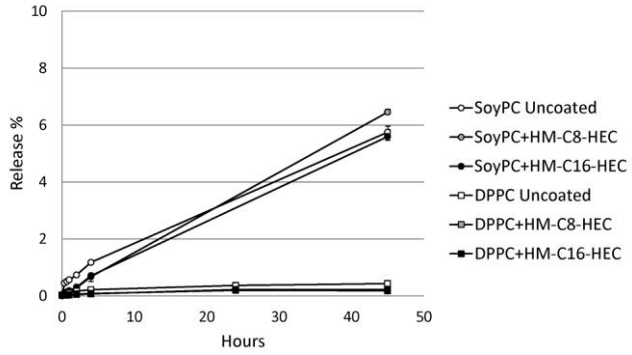

Figure 6

The release from the SoyPC formulations was significantly higher. The higher release from the SoyPC liposomes compared to the DPPC liposomes is attributed to the fluid state of the lipid membrane. At a temperature of 35 °C, the DPPC liposomes are still in the gel phase while the SoyPC liposomes are in the fluid phase (T_c +41.5 and -15±5 °C for DPPC and SoyPC, respectively [62]), and it is well known that the permeability of the gel phase lipid membrane is lower than the more flexible fluid state [63].

When liposomes are coated by a hydrophobically modified polymer, the polymer is believed to be anchored to the liposome surface by insertion of the hydrophobic groups into the liposome membrane [32-36]. Insertion of hydrophobic groups with different chain length could be expected to influence the release properties differently. However, no differences in release between the HM-C8-HEC and the HM-C16-HEC coated liposomes were detected, neither for the SoyPC nor the DPPC liposomes (Figure 6). Insertion of hydrophobic groups of different chain length into the liposome membrane could also be expected to affect the T_c of the liposomes differently. Changes in T_c can normally be detected by DSC [59]. Since the hydrophobic groups of HM-C16-HEC are of the same

length as in the fatty acid moiety of DPPC (C16:0), we expected it to be difficult to see any changes in T_c when coating with HM-C16-HEC. Therefore, mixtures of dimyristoyl phosphatidylcholine (fatty acid chain length C14:0) and HM-C8-HEC and HM-C16-HEC were tested, but no effects on the T_c were detected by DSC (data not shown). Also, dioleyl phosphatidylcholine (fatty acid chain length C18:1) liposomes mixed with both HM-C8-HEC and HM-C16-HEC were tested without detecting any effects. This is in line with what has been reported by Chieng and Chen, who coated DPPC liposomes with commercially available HM-HEC without detecting any effects on the T_c by DSC [34]. In a paper by Michel et al. [50] it is described how changes in the count rate during size measurements by DLS could be used for determination of the phase transition temperature of liposomes. Also this method was tried in the present paper, but no differences were seen for the different DPPC formulations. DPPC liposomes coated with HM-C8-HEC and HM-C16-HEC showed a steep drop in the derived count rate at exactly the same temperature as the T_c for the uncoated DPPC liposomes (41.5 °C [62], Figure 7), indicating no change in the membrane phase when coating the liposomes with the hydrophobically modified polymers.

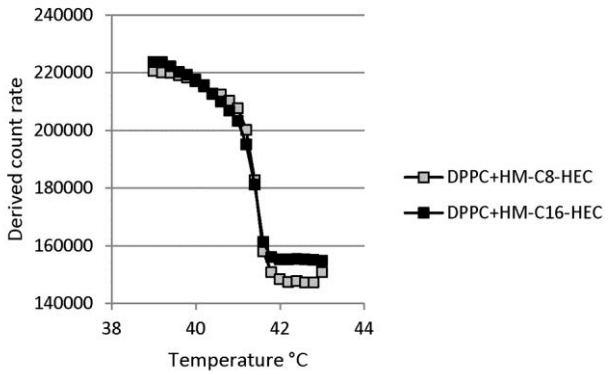

Figure 7

The absence of an effect on the T_c and on the release rate may be explained by the very low concentration of the hydrophobic groups in the hydrophobically modified polymers (1 mole%). The number of hydrophobic groups inserted into the membrane is expected to be small [34] and may be

too low to affect the T_c or release rate sufficiently to be detected by the analytical methods used. An additional explanation could be that C8 and C16 are too similar to affect the properties differently. Differences in release rate at 37 °C depending on degree of hydrophobic modification have, in fact, been shown for cholesterol succinyl chitosan coated liposomes [39]. However, in those studies the degree of hydrophobic modification was much higher (2.8-8 mole%) and consequently more HM-groups were expected to interact with the membrane. Also, they used another hydrophobic anchor and another marker, which both may influence the release properties.

Conclusion

Neutrally charged liposomes were successfully coated with hydrophobically modified HEC (containing 1 mole% of hydrophobic groups), whereas 2 mole% of hydrophobic groups was too high and did not result in acceptable products. The DPPC liposomes were stabilized by the polymer coating. No differences between the stability (size, zeta potential and leakage) could be detected after 24 weeks of storage at 4 °C between the HM-C8-HEC and HM-C16-HEC coated DPPC liposomes, and no differences in release properties at 35 °C. For the SoyPC liposomes, both HM-C8-HEC and HM-C16-HEC coated liposomes were stable for 12 weeks. However, after 24 weeks of storage the sizes of all the SoyPC formulations had increased significantly. The largest increase was observed for the HM-C8-HEC coated liposomes. This was probably due to the fluid lipid membrane and the relatively short hydrophobic anchoring group resulting in partly detaching of the polymer and bridging between the liposomes or interactions with other loose polymer chains. Thus, the most stable formulations were the HM-C8-HEC and the HM-C16-HEC coated DPPC liposomes. Since a hydrophilic uncharged surface has been reported as favourable for penetration deep into mucosa [30, 31] these formulations could be promising candidates for mucosal delivery and should be investigated further for mucoadhesion and release properties in biological fluids.

Acknowledgement

The authors want to thank Tove Larsen for technical assistance

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Jia X, Wang W, Han Q, Wang Z, Jia Y, Hu Z: **Micromixer Based Preparation of Functionalized Liposomes and Targeting Drug Delivery**. *ACS Medicinal Chemistry Letters* 2016, **7**(4):429-434.
2. Lyass O, Uziely B, Ben-Yosef R, Tzemach D, Heshing NI, Lotem M, Brufman G, Gabizon A: **Correlation of toxicity with pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in metastatic breast carcinoma**. *Cancer* 2000, **89**(5):1037-1047.
3. Paliwal SR, Paliwal R, Agrawal GP, Vyas SP: **Hyaluronic acid modified pH-sensitive liposomes for targeted intracellular delivery of doxorubicin**. *J Liposome Res* 2016, **26**(4):276-287.
4. Nunes PS, Rabelo AS, Souza JCCd, Santana BV, da Silva TMM, Serafini MR, dos Passos Menezes P, dos Santos Lima B, Cardoso JC, Alves JCS *et al*: **Gelatin-based membrane containing usnic acid-loaded liposome improves dermal burn healing in a porcine model**. *Int J Pharm* 2016, **513**(1–2):473-482.
5. Abd El Azim H, Nafee N, Ramadan A, Khalafallah N: **Liposomal buccal mucoadhesive film for improved delivery and permeation of water-soluble vitamins**. *Int J Pharm* 2015, **488**(1-2):78-85.
6. Joraholmen MW, Skalko-Basnet N, Acharya G, Basnet P: **Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections**. *European Journal of Pharmaceutical Sciences* 2015, **79**:112-121.
7. Grit M, Crommelin DJA: **The effect of aging on the physical stability of liposome dispersions**. *Chem Phys Lipids* 1992, **62**(2):113-122.
8. Grit M, Crommelin JA: **Chemical stability of liposomes - implications for their physical stability**. *Chem Phys Lipids* 1993, **64**(1-3):3-18.
9. Poste G, Bucana C, Raz A, Bugelski P, Kirsh R, Fidler IJ: **Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery**. *Cancer Res* 1982, **42**(4):1412-1422.
10. Rinaudo M, Quemeneur F, Pepin-Donat B: **Experimental characterization of liposomes stabilized by polyelectrolytes and mechanism of interaction involved**. *International Journal of Polymer Analysis and Characterization* 2012, **17**(1):1-10.
11. Smistad G, Bøyum S, Alund SJ, Samuelsen ABC, Hiorth M: **The potential of pectin as a stabilizer for liposomal drug delivery systems**. *Carbohydrate Polymers* 2012, **90**(3):1337-1344.
12. Takeuchi H, Kojima H, Yamamoto H, Kawashima Y: **Evaluation of circulation profiles of liposomes coated with hydrophilic polymers having different molecular weights in rats**. *J Control Release* 2001, **75**(1-2):83-91.
13. Henriksen I, Vagen SR, Sande SA, Smistad G, Karlsen J: **Interactions between liposomes and chitosan .2. Effect of selected parameters on aggregation and leakage**. *Int J Pharm* 1997, **146**(2):193-203.
14. Laye C, McClements DJ, Weiss J: **Formation of biopolymer-coated liposomes by electrostatic deposition of chitosan**. *J Food Sci* 2008, **73**(5):N7-N15.
15. Nguyen S, Alund SJ, Hiorth M, Kjøniksen A-L, Smistad G: **Studies on pectin coating of liposomes for drug delivery**. *Colloids and Surfaces B: Biointerfaces* 2011, **88**(2):664-673.

16. Alund SJ, Smistad G, Hiorth M: **A multivariate analysis investigating different factors important for the interaction between liposomes and pectin**. *Colloid Surf A-Physicochem Eng Asp* 2013, **420**:1-9.
17. Klemetsrud T, Hiorth M, Smistad G, Kjøniksen AL: **Characterization of temperature induced changes in liposomes coated with poly(N-isopropylacrylamide-co-methacrylic acid)**. *J Colloid Interface Sci* 2015, **450**:7-16.
18. Pistone S, Rykke M, Smistad G, Hiorth M: **Polysaccharide-coated liposomal formulations for dental targeting**. *Int J Pharm* 2017, **516**(1–2):106-115.
19. Andersen T, Bleher S, Flaten GE, Tho I, Mattsson S, Skalko-Basnet N: **Chitosan in Mucoadhesive Drug Delivery: Focus on Local Vaginal Therapy**. *Marine Drugs* 2015, **13**(1):222-236.
20. Joraholmen MW, Vanic Z, Tho I, Skalko-Basnet N: **Chitosan-coated liposomes for topical vaginal therapy: Assuring localized drug effect**. *Int J Pharm* 2014, **472**(1-2):94-101.
21. Karn PR, Vanic Z, Pepic I, Skalko-Basnet N: **Mucoadhesive liposomal delivery systems: the choice of coating material**. *Drug Dev Ind Pharm* 2011, **37**(4):482-488.
22. Hao WJ, Xia T, Shang YZ, Xu SH, Liu HL: **Characterization and release kinetics of liposomes inserted by pH-responsive bola-polymer**. *Colloid and Polymer Science* 2016, **294**(7):1107-1116.
23. Pippa N, Meristoudi A, Pispas S, Demetzos C: **Temperature-dependent drug release from DPPC:C12H25-PNIPAM-COOH liposomes: Control of the drug loading/release by modulation of the nanocarriers' components**. *Int J Pharm* 2015, **485**(1–2):374-382.
24. Mosqueira VCF, Legrand P, Pinto-Alphandary H, Puisieux F, Barratt G: **Poly(D,L-lactide) nanocapsules prepared by a solvent displacement process: Influence of the composition on physicochemical and structural properties**. *J Pharm Sci* 2000, **89**(5):614-626.
25. Ney P: **Zetapotentiale und flotierbarkeit von mineralen**. New York: Springer; 1973.
26. Nguyen S, Hiorth M, Rykke M, Smistad G: **Polymer coated liposomes for dental drug delivery - Interactions with parotid saliva and dental enamel**. *European Journal of Pharmaceutical Sciences* 2013, **50**(1):78-85.
27. Klemetsrud T, Kjøniksen A-L, Hiorth M, Jacobsen J, Smistad G: **Polymer coated liposomes for use in the oral cavity – a study of the in vitro toxicity, effect on cell permeability and interaction with mucin**. *J Liposome Res* 2016:1-12.
28. Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov AA, Trubetskoy VS, Herron JN, Gentry CA: **Poly(ethylene glycol) on the liposome surface - on the mechanism of polymer-coated liposome longevity**. *Biochim Biophys Acta-Biomembr* 1994, **1195**(1):11-20.
29. Senior J, Delgado C, Fisher D, Tilcock C, Gregoriadis G: **Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: Studies with poly(ethylene glycol)-coated vesicles**. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1991, **1062**(1):77-82.
30. Lai SK, Wang YY, Hanes J: **Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues**. *Advanced Drug Delivery Reviews* 2009, **61**(2):158-171.
31. Wang YY, Lai SK, Suk JS, Pace A, Cone R, Hanes J: **Addressing the PEG mucoadhesivity paradox to engineer nanoparticles that "slip" through the human mucus barrier**. *Angewandte Chemie - International Edition* 2008, **47**(50):9726-9729.
32. Ringsdorf H, Sackmann E, Simon J, Winnik FM: **Interactions of liposomes and hydrophobically-modified poly-(N-isopropylacrylamides): an attempt to model the cytoskeleton**. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1993, **1153**(2):335-344.
33. Thongborisute J, Takeuchi H, Yamamoto H, Kawashima Y: **Properties of liposomes coated with hydrophobically modified chitosan in oral liposomal drug delivery**. *Pharmazie* 2006, **61**(2):106-111.
34. Chieng YY, Chen SB: **Rheological study of hydrophobically modified hydroxyethyl cellulose and phospholipid vesicles**. *J Colloid Interface Sci* 2010, **349**(1):236-245.

35. Polozova A, Winnik FM: **Mechanism of the interaction of hydrophobically-modified poly-(N-isopropylacrylamides) with liposomes**. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1997, **1326**(2):213-224.
36. Bai G, Nichifor M, Bastos M: **Association and Phase Behavior of Cholic Acid-Modified Dextran and Phosphatidylcholine Liposomes**. *Journal of Physical Chemistry Letters* 2010, **1**(6):932-936.
37. Bai GY, Wang YJ, Nichifor M, Bastos M: **Critical Role of the Degree of Substitution in the Interaction of Biocompatible Cholic Acid-Modified Dextrans with Phosphatidylcholine Liposomes**. *Langmuir* 2013, **29**(43):13258-13268.
38. Sunamoto J, Sato T, Taguchi T, Hamazaki H: **Naturally occurring polysaccharide derivatives which behave as an artificial cell wall on an artificial cell liposome**. *Macromolecules* 1992, **25**(21):5665-5670.
39. Wang YS, Tu SL, Li RS, Yang XOY, Liu LR, Zhang QQ: **Cholesterol succinyl chitosan anchored liposomes: preparation, characterization, physical stability, and drug release behavior**. *Nanomedicine-Nanotechnology Biology and Medicine* 2010, **6**(3):471-477.
40. Hong YJ, Kim JC: **pH- and Calcium Ion-Dependent Release from Egg Phosphatidylcholine Liposomes Incorporating Hydrophobically Modified Alginate**. *Journal of Nanoscience and Nanotechnology* 2010, **10**(12):8380-8386.
41. Auguste DT, Prud'homme RK, Ahl PL, Meers P, Kohn J: **Association of hydrophobically-modified poly(ethylene glycol) with fusogenic liposomes**. *Biochim Biophys Acta-Biomembr* 2003, **1616**(2):184-195.
42. Matuszewska B, Keogan M, Fisher DM, Soper KA, Hoe CM, Huber AC, Bondi JV: **Acidic fibroblast growth factor: Evaluation of topical formulations in a diabetic mouse wound healing model**. *Pharmaceutical Research* 1994, **11**(1):65-71.
43. Gandhi R, Kaul CL, Panchagnula R: **Extrusion and spheronization in the development of oral controlled-release dosage forms**. *Pharmaceutical Science & Technology Today* 1999, **2**(4):160-170.
44. Anders R, Merkle HP: **Evaluation of laminated muco-adhesive patches for buccal drug delivery**. *Int J Pharm* 1989, **49**(3):231-240.
45. Meland H-G, Røv-Johnsen A, Smistad G, Hiorth M: **Studies on surface coating of phospholipid vesicles with a non-ionic polymer**. *Colloids and Surfaces B: Biointerfaces* 2014, **114**:45-52.
46. Beheshti N, Zhu KZ, Kjoniksen AL, Nystrom B: **Interaction behaviors in aqueous solutions of negatively and positively charged hydrophobically modified hydroxyethylcellulose in the presence of an anionic surfactant**. *Colloid Surf A-Physicochem Eng Asp* 2008, **328**(1-3):79-89.
47. Beheshti N, Bu H, Zhu KZ, Kjoniksen AL, Knudsen KD, Pamies R, Cifre JGH, de la Torre JG, Nystrom B: **Characterization of interactions in aqueous solutions of hydroxyethylcellulose and its hydrophobically modified analogue in the presence of a cyclodextrin derivative**. *J Phys Chem B* 2006, **110**(13):6601-6608.
48. Miyajima T KT, Kita K, Kamitani H, Yamaki K: **Polysaccharide derivative, and preparation process and use thereof**. In: *US Patent 5, 891, 450.* 1999.
49. Beheshti N, Zhu K, Kjoniksen AL, Nystrom B: **Effects of beta-cyclodextrin and beta-cyclodextrin polymer addition and temperature on the modulation of hydrophobic interactions in aqueous solutions of two hydrophobically modified biopolymers**. *Journal of Non-Crystalline Solids* 2007, **353**(41-43):3906-3914.
50. Michel N, Fabiano A-S, Polidori A, Jack R, Pucci B: **Determination of phase transition temperatures of lipids by light scattering**. *Chem Phys Lipids* 2006, **139**(1):11-19.
51. Tanaka R, Meadows J, Phillips GO, Williams PA: **Viscometric and spectroscopic studies on the solution behaviour of hydrophobically modified cellulosic polymers**. *Carbohydrate Polymers* 1990, **12**(4):443-459.

52. Gelman RA, Barth HG: **Viscosity Studies of Hydrophobically Modified (Hydroxyethyl)cellulose**. In: *Water-Soluble Polymers. Volume 213*, edn.: American Chemical Society; 1986: 101-110.
53. Stensrud G, Smistad G, Agren TL, Sande SA, Karlsen J: **Effects of gamma irradiation on the physical stability of liposomal phospholipids**. *J Liposome Res* 1997, **7**(4):503-528.
54. Stępniewski M, Bunker A, Pasenkiewicz-Gierula M, Karttunen M, Róg T: **Effects of the Lipid Bilayer Phase State on the Water Membrane Interface**. *The Journal of Physical Chemistry B* 2010, **114**(36):11784-11792.
55. Essmann U, Perera L, Berkowitz ML: **The Origin of the Hydration Interaction of Lipid Bilayers from MD Simulation of Dipalmitoylphosphatidylcholine Membranes in Gel and Liquid Crystalline Phases**. *Langmuir* 1995, **11**(11):4519-4531.
56. McIntosh TJ, Simon SA: **Hydration and steric pressure between phospholipid bilayers**. *Annual Review of Biophysics and Biomolecular Structure* 1994, **23**:27-51.
57. Frederik PM, Stuart MCA, Bomans PHH, Busing WM, Burger KNJ, Verkleij AJ: **Perspective and limitations of cryo-electron microscopy. From model systems to biological specimens**. *Journal of Microscopy-Oxford* 1991, **161**:253-262.
58. Adamczak MI, Hagesaether E, Smistad G, Hiorth M: **An in vitro study of mucoadhesion and biocompatibility of polymer coated liposomes on HT29-MTX mucus-producing cells**. *Int J Pharm* 2016, **498**(1-2):225-233.
59. New RRC: **Liposomes a practical approach**. Oxford, U.K.: IRL Press; 1990.
60. Kepczynski M, Jamróz D, Wytrwal M, Bednar J, Rzad E, Nowakowska M: **Interactions of a Hydrophobically Modified Polycation with Zwitterionic Lipid Membranes**. *Langmuir* 2012, **28**(1):676-688.
61. Volchansky A, Cleaton-Jones P: **Variations in oral temperature**. *Journal of Oral Rehabilitation* 1994, **21**(5):605-611.
62. Cevc G: **Phospholipids Handbook**. New York: Marcel Dekker; 1993.
63. Carruthers A, Melchior DL: **Studies of the relationship between bilayer water permeability and bilayer physical state**. *Biochemistry* 1983, **22**(25):5797-5807.

Figure captions

Figure 1 – Representative samples of EggPC liposomes coated with 0.1% (w/w) solution of (a) and (d) HM-C8-HEC, (b) HM-C12-HEC, (c) and (f) HM-C16-HEC, and (e) uncoated liposomes. The mole% given is the amount of hydrophobic groups attached to the polymer. The correlation functions of 3 runs in the same cuvette are shown for all samples. The transmittance (T%) is the average of 3 separate samples (±SD).

Figure 2 – Average diameter (black curve) and PDI values (grey curve) of DPPC liposomes in the temperature range 25 – 45 °C. The scanning direction was from high to lower temperature with a scanning rate of 0.2 degrees pr. min. Equilibration time at each temperature step was 60 s and three replicates were run at each step

Figure 3 – Average diameter (a) and PDI values (b) of the samples during storage at 4 °C. The error bars represent the highest and lowest values (n = 3). The points without error bars have differences equal to or smaller than the size of the symbols.

Figure 4 – The average zeta potential of the samples during storage at 4 °C. The error bars represent the highest and lowest value (n=3). The points without error bars have differences equal to or smaller than the size of the symbols.

Figure 5 - The leakage of encapsulated marker (CF) during storage at 4 °C. The error bars represent the highest and lowest value (n=3). The points without error bars have differences equal to or smaller than the size of the symbols.

Figure 6 – The release of encapsulated marker (CF) at 35 °C. Error bars representing the highest and lowest value are included (n=3) but not visible; the differences are smaller than the size of the symbols.

Figure 7 – Effect of changing the temperature on the derived count rate during the size measurement of HM-HEC coated liposomes.